LFA-1/ICAM-1 Interaction Lowers the Threshold of B Cell Activation by Facilitating B Cell Adhesion and Synapse Formation

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Summary

The integrin LFA-1 and its ligand ICAM-1 mediate B cell adhesion, but their role in membrane-bound antigen recognition is still unknown. Here, using planar lipid bilayers and cells expressing ICAM-1 fused to green fluorescence protein, we found that the engagement of B cell receptor (BCR) promotes B cell adhesion by an LFA-1-mediated mechanism. LFA-1 is recruited to form a mature B cell synapse segregating into a ring around the BCR. This distribution is maintained over a wide range of BCR/antigen affinities (10^6 M^{-1} to 10^{11} M^{-1}). Furthermore, the LFA-1 binding to ICAM-1 reduces the level of antigen required to form the synapse and trigger a B cell. Thus, LFA-1/ICAM-1 interaction lowers the threshold for B cell activation by promoting B cell adhesion and synapse formation.

Introduction

B cell activation is triggered by antigen recognition through the B cell receptor (BCR). BCR engagement initiates intracellular signaling cascades that result in B cell proliferation and differentiation (Benschop and Cambier, 1999; Reth et al., 2000). A second role for the BCR is antigen uptake which leads to the generation of antigen-derived peptides loaded on MHC class II molecules for presentation to T cells (Lanzavecchia, 1985; Rock et al., 1984). Both BCR functions seem to be interdependent, as BCR signaling is necessary for the correct and rapid targeting of the antigen to the MHC class II-containing compartments (Wagle et al., 2000).

While B cells do respond to soluble antigen, membrane-associated antigens are particularly effective in promoting B cell activation. At early stages of differentiation, encounters with low-affinity membrane self-antigens induce B cell deletion (Lang et al., 1996), whereas recognition of high-affinity soluble self-antigens leads to B cell anergy, but not deletion (Goodnow et al., 1988). In addition, during the course of an immune response,

soluble antigens tethered on cell surfaces by Fc or complement receptors in the form of immunocomplex (IC) are likely to be the main form of antigen encountered by a B cell (reviewed in Haberman and Shlomchik, 2003; Kosco-Vilbois, 2003). It has been suggested that B cells are able to recognize antigen presented by dendritic cells (Wykes et al., 1998). Furthermore, in the germinal centers, follicular dendritic cells (FDCs) have been shown to play an important role in the retention of intact antigen in the form of IC that is then presented to B cells (Szakal et al., 1988). Finally, membrane proteins, hormone receptors, and lipids have been identified as autoantigens (Lernmark, 2001). However, it is still unknown how B cells become activated by these membrane antigens. Thus, membrane antigen recognition is likely to play an important role in determining the fate of B cells in vivo during an immune response, as well as in establishing tolerance.

We recently showed that B cell recognition of membrane antigen is accompanied by the formation of an immunological synapse between the B cell and antigen-presenting cells (APCs) (Batista et al., 2001). The immunological synapse was initially defined as a specialized contact between lymphocytes and their targets in which different receptors segregate into defined domains known as supramolecular activation clusters (SMACs) (Dustin et al., 1998; Grakoui et al., 1999; Krummel et al., 2000; Monks et al., 1998). The T cell receptor aggregates into a central SMAC (cSMAC) surrounded by a peripheral ring of LFA-1 adhesion molecules (peripheral SMAC, pSMAC). While a similar distribution into cSMAC and pSMAC has been observed in CD4$^+$ and CD8$^+$ T cells (Potter et al., 2001; Stinchcombe et al., 2001), NK inhibition has been shown to be associated with an inverse pattern (Davis et al., 1999). While certain receptors, such as CD28 (Bromley et al., 2001), localize to the cSMAC, others, such as CD45 or CD43, have been shown to be excluded from the synapse altogether (Allenspach et al., 2001; Delon et al., 2001; Freiberg et al., 2002; Johnson et al., 2000; Leupin et al., 2000). The B cell synapse shares some of these aspects, such as the central clustering of the BCR in the area of B cell-APC contact and the exclusion of CD45 (Batista et al., 2001). However, the role of LFA-1 or the existence of a pSMAC in the B cell synapse formation has never been addressed.

LFA-1 (CD11a/CD18) belongs to the integrin family of cell adhesion molecules and is expressed on all leukocytes including B cells, where increased levels of expression and adhesion have also been observed upon BCR crosslinking (Dang and Rock, 1991). Its main ligand, ICAM-1 (CD54), is expressed on a wide range of cell lineages (leukocytes, FDCs, DCs, endothelium) (Springer, 1990). LFA-1/ICAM-1 has been implicated in the physical interaction of B cells with DC and FDCs (Koopman et al., 1991; Kushnir et al., 1998). Furthermore, interaction of this integrin pair has been shown to prevent apoptosis of germinal center B cells (Koopman et al., 1994). More recently, they have also been shown to play a critical role in the compartmentalization of B cells into peripheral lymphoid tissue (Lo et al., 2003; Lu and Cyster, 2002).

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This evidence implicates LFA-1/ICAM-1 in several aspects of the B cell biology.

To initiate an immune response, naive B cells are likely to respond to antigens through low-affinity interactions. However, upon antigen encounter, variants with considerably higher affinity are generated and preferentially selected by affinity maturation (Foote and Milstein, 1991; Moller, 1987). Therefore, unlike T or NK cells, which recognize antigen with rather low affinity (Matsui et al., 1991; Sykulev et al., 1994), in vivo B cells must be able to sense an exceptionally wide range of different affinities. In recent years we and others have performed a series of studies to explore how the B cell response varies according to antigen affinity. These studies have shown that the B cell response is critically dependent on the affinity of the BCR for the antigen (Batista and Neuberger, 1998; Kouskoff et al., 1998; Shih et al., 2002a, 2002b). The wide range of affinities recognized by B cells makes them unique for studying synapse formation.

In the present study we have analyzed the contribution of the LFA-1/ICAM-1 interaction during B cell antigen recognition and B cell activation. The results show that BCR engagement promotes B cell attachment to ICAM-1 by an LFA-1-dependent mechanism. LFA-1 is recruited to form a peripheral ring (pSMAC), which surrounds the central cluster of BCR/antigen (cSMAC) demonstrating that a pSMAC forms in the B cell synapse. This pattern is maintained over a wide range of antigen affinities. Interestingly, the LFA-1/ICAM-1 interaction increases the adhesion of the B cells under conditions of limited antigen, therefore lowering the amount of antigen required for synapse formation and B cell activation. This quantitative synergy of the BCR with adhesion mechanisms highlights an additional advantage of membrane-associated antigen recognition.

Results

Peripheral Distribution of LFA-1/ICAM-1 in the B Cell Synapse: Formation of a Docking Structure

In order to examine the role of LFA-1/ICAM-1 during membrane antigen recognition, we analyzed the interaction of naive 3-83 B cells (Russell et al., 1991) with L cells expressing GFP-ICAM-1 as antigen-presenting cells. We chose cells expressing H-2K^K, since this molecule is recognized with high affinity by the 3-83 BCR and constitutes an antigen for these transgenic B cells. To directly assess the localization of the BCR, B cells were stained with nonblocking, fluorescently labeled Fab fragments against IgM. Confocal microscopy revealed that, within the first minute of contact, the BCR polarizes toward the APC while ICAM-1 is restricted to the periphery (pSMAC), forming a mature immunological synapse (Figure 1A and see Supplemental Movie S1A at http://www.immunity.com/cgi/content/full/20/5/589/DC1). Surprisingly, three-dimensional reconstruction showed that, in 90% of the cells, the BCR and ICAM-1 are contained in a chalice-like three-dimensional docking structure. The BCR is concentrated into the center of this chalice-like structure, but in contrast to the T cell immunological synapse, which is characterized by a flat interface, the ICAM-1 is born on multiple projections of
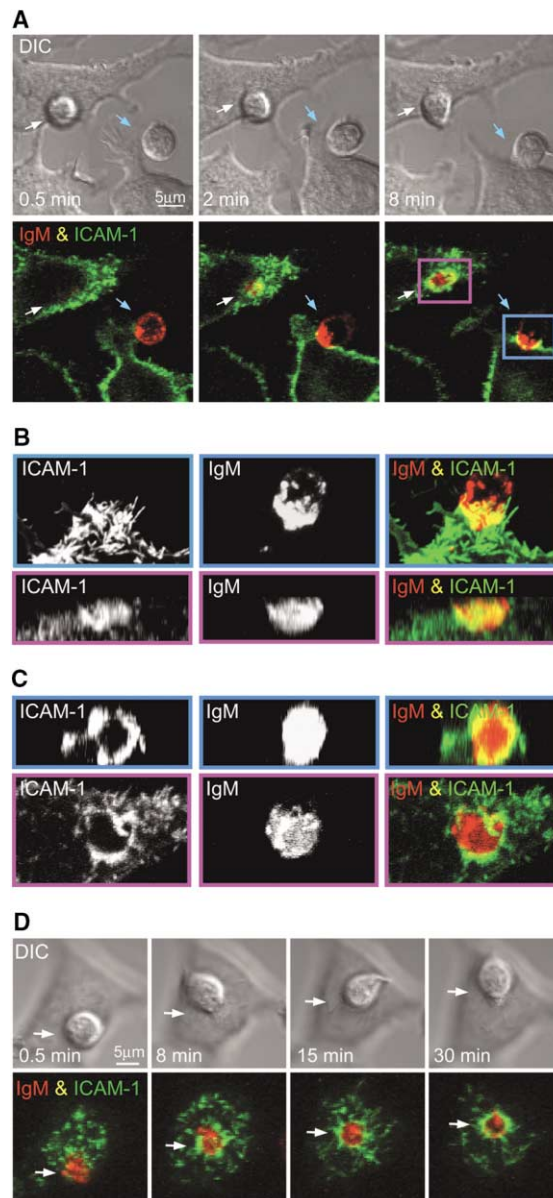

Figure 1. ICAM-1 Is Recruited in the B Cell Synapse, Forming a Docking Structure

3-83 B cells stained with Cy5-conjugated nonblocking Fabs anti-IgM (red) were analyzed in the presence of APCs expressing ICAM-1-GFP (green) by confocal microscopy.

(A) Time-lapse fluorescent images represent a merged projection of several horizontal confocal sections at the specified times. DIC images correspond to one representative horizontal section.

(B and C) Side and top views, respectively, of the 3D reconstruction of the docking structure formed by cells in (A). Separate and merged fluorescence images of ICAM-1 and IgM are shown for the B cells indicated by arrows and colored frames at the last time point.

(D) Dynamic features of the B cell synapse. Time-lapse confocal microscopy of 3-83 B cells, previously stained with Cy5-conjugated nonblocking Fabs anti-IgM (red), in contact with ICAM-GFP (green) expressing APCs. Panels representing the DIC image and a projection of several confocal sections at the specified times are shown. See Supplemental Movie S1 at http://www.immunity.com/cgi/content/full/20/5/589/DC1 for complete time course of different examples and 3D rotations of the docking structure. Supplemental Movie S3 shows different examples of moving synapses assembled into a movie.

Table 1. Antigen Affinities

Antigen	$K_{on} \times 10^3$ (M^{-1} sec^{-1})	K_{off} (sec^{-1})	$K_A \times 10^6$ (M^{-1})
3-83 Binding			
p31	10	1.4×10^{-4}	65
p11	8.5	1.2×10^{-3}	7
p5	ND	ND	>1
p0	—	—	—
HyHEL10 Binding			
HEL^{WT}	2000	4×10^{-5}	50,000
HEL^{RDGN}	2000	4×10^{-3}	500
HEL^{KD}	$\sim$2000	6×10^{-2}	30
HEL^{RKD}	$\sim$2000	$\sim$3 $\times 10^{-1}$	$\sim$3

The derivation of the HEL mutants and references for the affinity determinations for wild-type lysozymes are provided in Batista and Neuberger (1998) and the Experimental Procedures. The mutations described that diminish HyHEL10 binding have no effect on the affinity for F10. The K_A values for the different peptides/3-83 antibody were determined using the Biacore as described in the Experimental Procedures; repeat estimates were within 20% except for the p5, owing to its low affinity. Due to the way in which the affinity measurements were performed in the case of 3.83, the values derived represent the affinity of the dimeric antibody for the peptide. In the case of HyHEL10, they account for the monomeric interaction of this antibody for the lysozymes.

the L cell membrane that cup the B cell (Figures 1B and 1C and Supplemental Movie S1B). Thus, these results reveal the formation of a pSMAC containing ICAM-1 in the B cell synapse with a unique three-dimensional structure.

To further characterize the interaction of B cells with APCs, we performed video microscopy experiments using 3-83 B cells or control wild-type B cells, which do not recognize antigen on the surface of the L cells. Quantification of the number of cells bound to the APCs shows that both control B cells and 3-83 B cells formed high numbers of conjugates with their targets (Supplemental Movie S2). Similar numbers of conjugates were also observed in the absence of ICAM-1 or LFA-1, indicating that other accessory molecules may be involved in promoting this interaction. However, while the interaction of a control B cell with the same target cell was short term, once a 3-83 B cell attached to its H-2K^K target it remained bound for at least 1 hr. Interestingly, during this long-lasting interaction, 10%–20% of the 3.83 B cells appeared to be able to move over the surface of the APC. Time-lapse confocal microscopy and three-dimensional reconstruction revealed that synapses were retained during this movement (Figure 1D and Supplemental Movie S3). It is not clear whether this movement is a result of B cell amoeboid movement or transport of the B cell by the cytoskeleton of the L cell or both. These results indicate that, upon forming a synapse, a B cell can move over the surface of an APC for several tens of microns without altering the BCR and ICAM-1 distribution in the cSMAC and pSMAC, respectively.

Real-Time Kinetics of Mature B Cell Synapse Formation

To extend our findings on the molecular aspects of the B cell synapse, we precisely quantified the segregation and accumulation of antigen and ICAM-1 in real time. This was carried out by replacing APCs with glass-supported planar lipid bilayers containing a fluorescently labeled version of ICAM-1 and antigen. Purified naive B cells from 3-83 mice were settled onto bilayers containing Alexa 543-conjugated GPI-linked ICAM-1 and p31 biotinylated peptide as antigen, which binds the 3-83 BCR with an affinity 6.5×10^7 M^{-1} (Table 1; Kouskoff

et al., 1998). The peptide was tethered to the surface of the lipid bilayers through Alexa 488-conjugated avidin linked to biotinylated lipids. The pattern of ICAM-1 and antigen distribution over time was followed by confocal microscopy, and the contact of B cells with the membrane was assessed by interference reflection microscopy (IRM). As soon as B cells touched the membrane, a close contact was made and antigen and ICAM-1 accumulation was detected (Figure 2A and Supplemental Movie S4). Similar to our results with APCs, ICAM-1 is very rapidly aggregated to form a peripheral ring (pSMAC) while antigen gathers into a central cluster (cSMAC). This pattern of mature synapse was stable for more than 1 hr (data not shown). Quantification of the amounts of antigen and ICAM-1 showed that a maximal recruitment of both molecules was reached within 5 min and a plateau after 10 min (Figure 2B). These results suggested that, during B cell synapse formation, antigen and ICAM-1 molecules are engaged upon initial contact and then aggregated rapidly into a fully formed cSMAC and pSMAC, respectively.

The B Cell Synapse Is Maintained over a Wide Range of Antigen Densities and Affinities

To investigate how the pattern of the mature synapse may be influenced by antigen density, we followed the interaction of B cells with lipid bilayers carrying a constant ICAM-1 concentration but decreasing densities of p31 antigen. As shown in Figure 3A, the distribution of BCR and ICAM-1 in the cSMAC and pSMAC, respectively, is preserved over a range of antigen densities. Similar amounts of ICAM-1 are segregated in the periphery of the synapse at all antigen densities (Figure 3B). Whereas p31 antigen density may be reduced to 5 molecules per μm^2 and still be efficiently gathered by B cells, no synapse or membrane contact could be observed below this antigen concentration (data not shown). These results indicate that a minimum density of antigen is required for ICAM-1 redistribution but that increasing the antigen concentration above this minimum allows no further ICAM-1 recruitment.

To probe the effect of varying antigen/BCR binding strength on the pattern of receptor distribution during
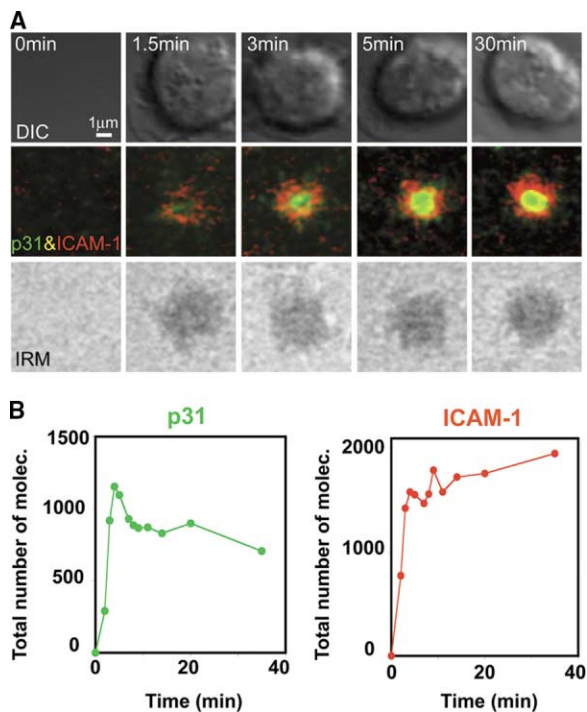
A

B

Figure 2. Real-Time Quantification of Antigen and ICAM-1 Recruitment to B Cell Synapse

Naive 3-83 B cells were settled onto planar lipid bilayers containing GPI-linked ICAM-1 (red) at 170 mole/μm^2 and p31 (green) antigen at 50 mole/μm^2.

(A) Central panels show the accumulation of the antigen p31 (green) and ICAM-1 (red) in the pattern of a mature synapse at the specified time points. Top and bottom panels show DIC and IRM images of the same time points. See Supplemental Movie S4 for complete time course of fluorescence and IRM images assembled into a movie.
(B) Quantification of the total number of molecules of p31 antigen and ICAM-1 during synapse formation.

synapse formation, we initially exploited a set of previously characterized peptides (p31, p11, p5, plus null peptide, p0, as a control), which bind the 3-83 BCR with decreasing affinities (Table 1). The pattern and level of antigen and ICAM-1 accumulation were evaluated. The p0, null peptide, did not induce any B cell interaction or ICAM-1 accumulation, even at high densities (Figure 3C). In contrast, p11 and p5 peptides with a reduced affinity for 3-83 BCR (Table 1) were able to form a mature synapse as defined by a central cluster of antigen aggregation and ICAM-1 peripheral distribution (Figure 3C). The recruitment of ICAM-1 was similar in all cases, as in the case of p31. However, the amount of antigen gathered was dependent on both the density and its affinity for the BCR. In the case of the low-affinity peptide, p5, mature synapses were observed with a very low frequency and only with extremely high densities of this peptide on the membrane (Figures 3C and 3D).

In order to extend our study over a wider range of antigen affinities, we used a second transgenic system based on the hen egg lysozyme (HEL) as an antigen. B cells from MD4 transgenic mice carry an IgM$^+$IgD$^+$ HEL-specific BCR (Goodnow et al., 1988). By challenging these transgenic B cells with a previously well-characterized panel of different HEL mutants (Table 1; Batista

and Neuberger, 1998), we were able to study the structure of the synapse within a unique range of physiological affinities (Table 1). To tether the lysozymes onto the bilayers, we used a monobiotinylated anti-HEL monoclonal, F10, as a bridge. As we have previously shown (Batista and Neuberger, 1998), this antibody does not compete with the binding of the MD4 BCR. As shown in Figure 3E, when MD4 B cells were settled onto these bilayers, the distribution of ICAM-1 and antigen into pSMAC and cSMAC was preserved independently of the strength of BCR/antigen interaction. Quantitative analysis revealed that similar quantities of ICAM-1 were recruited in all cases. In contrast, the amount of antigen gathered was dependent on its density and BCR affinity (Figure 3F). Similar results were obtained when D.13, a monoclonal antibody with a substantially lower affinity for the lysozymes, was used as a tethering bridge (Supplemental Movie S4B), indicating that high-affinity tethering is not essential for the formation of a mature synapse.

Our findings show that, for a B cell to form a mature synapse, the BCR needs to recognize antigen with an affinity higher than $10^6 \, M^{-1}$, but only if the latter is present at a high enough density. Thus, the amount of antigen gathered depends on its density and affinity for the BCR. However, the amount of ICAM-1 recruited and its segregation into a peripheral ring are independent of the strength of the BCR/antigen binding above this threshold.

The LFA-1/ICAM-1 Interaction Increases the Capacity of B Cells to Attach to Target Membranes, Lowering the Threshold of Antigen Density to Form a Synapse

To dissect the function of LFA-1/ICAM-1 during membrane antigen recognition, we initially evaluated whether this interaction may enhance the capacity of B cells to adhere to a target membrane. To this end, we compared the ability of naive B cells to attach to membranes in the presence or absence of antigen and/or ICAM-1. As judged by IRM, at high densities of p31, 100% of 3.83 B cells formed tight contacts, regardless of whether ICAM-1 was included in the bilayers. However, if the density of p31 was decreased, tight contacts with the target membrane were only observed in the presence of ICAM-1 (Figure 4A). In the absence of antigen, less than 10% of the B cells attached to the bilayers, indicating that BCR engagement was essential to trigger adhesion to ICAM-1. Quantitative examination of the frequency of B cells making contacts with membranes loaded with different affinity antigens is shown in Figure 4. This analysis revealed that, in the presence of ICAM-1, less antigen is needed to form a tight contact. However, it was noticeable that this capacity of ICAM-1 to increase B cell adhesion was substantially higher when the antigens encountered in the bilayers were of medium or low affinity. In these cases a 10-fold reduction in the amount of antigen required to make a tight contact was observed. Thus, ICAM-1 appears to play a critical role in promoting adhesion under limiting concentration of antigen, but this function seems to be regulated by BCR engagement.

To determine whether improved attachment to the target membrane had an effect on synapse formation, we analyzed the antigen and ICAM-1 distribution in the
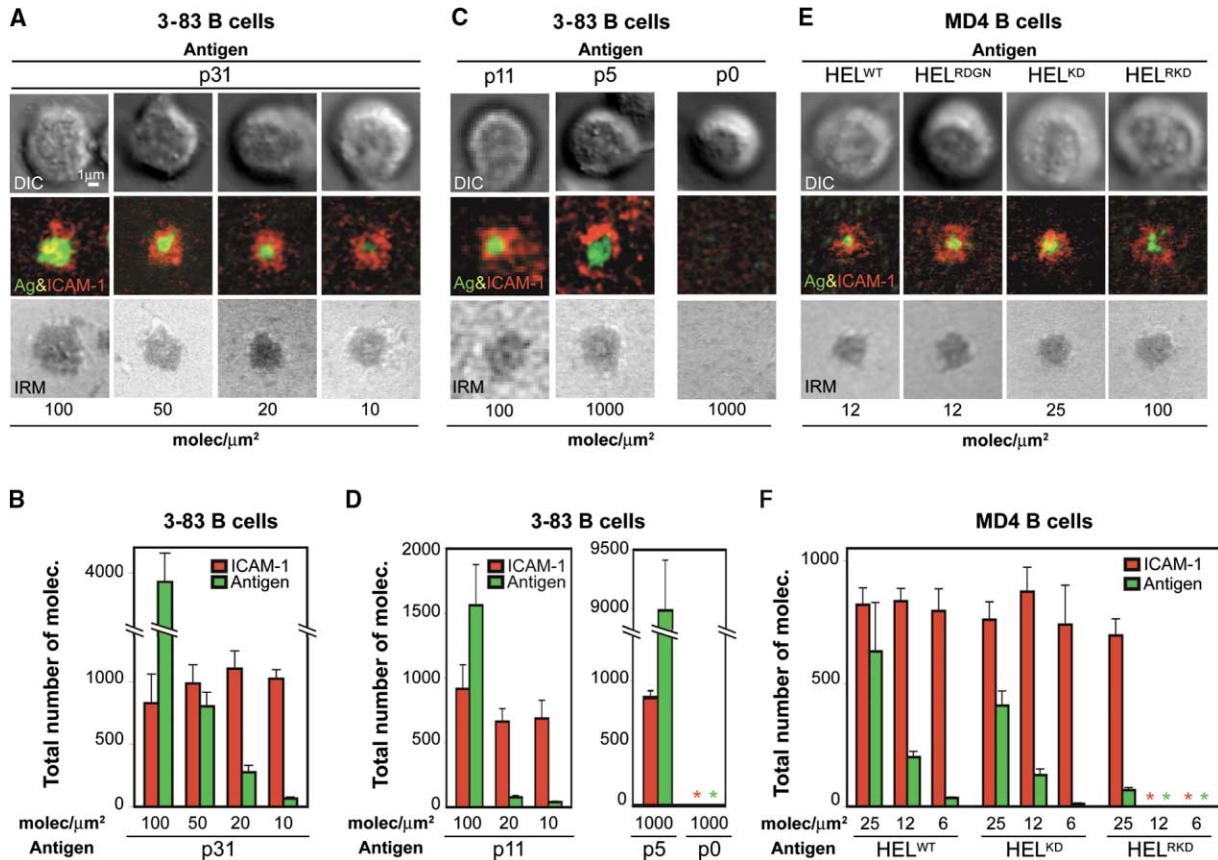
Figure 3. ICAM-1 Is Recruited in the B Cell Synapse over a Wide Range of Antigen Affinities and Densities

(A and C) 3-83 B cells were settled onto membranes containing constant amounts of ICAM-1 (170 mole/μm^2, in red) and different antigens (green). Fluorescence-merged, DIC, and IRM from a representative B cell are shown.

(A) Decreasing densities of p31.

(B) Quantification of the number of ICAM-1 and antigen molecules recruited in (A). Data represent the mean of 50 cells analyzed in each case.

(C) Images show a representative synapse obtained with p0 (null), p5 ($K_A \approx 0.1-1 \times 10^6$ M^{-1}), and p11 ($K_A \approx 7 \times 10^6$ M^{-1}).

(D) Quantification of the total number ICAM-1 and antigen molecules recruited for each of the affinities shown in (C). Data represent the mean of 50 cells analyzed in each case.

(E) Naive MD4 B cells were settled onto membranes containing constant amounts of ICAM-1 (170 mole/μm^2, in red) and different lyzosymes as antigens (green): HEL^{WT} ($K_A = 5 \times 10^{10}$ M^{-1}), HEL^{RDGN} ($K_A = 5 \times 10^8$ M^{-1}), HEL^{KD} ($K_A \approx 3 \times 10^7$ M^{-1}), HEL^{RKD} ($K_A \approx 3 \times 10^6$ M^{-1}). DIC, fluorescence-merged, and IRM images are shown.

(F) Quantification of the number of ICAM-1 and antigen molecules recruited in the B cell synapse shown in (E). Data represent the mean of 60 cells in each case.

range of antigen affinity and density where contact formation was ICAM-1 dependent. In the range of densities analyzed, a well-formed immunological synapse was observed whenever contacts were made, and no antigen accumulation was detected in the absence of contact formation as detected by IRM (Figure 5A). Thus, these results suggest that under conditions of limited antigen, the presence of ICAM-1 in the target membrane facilitates attachment of the B cell throughout the process of mature synapse formation.

It was important to ascertain whether the effects of ICAM-1 were indeed due to LFA-1 engagement on the B cell. 3-83 BCR transgenic mice could not be crossed into an LFA-1 knockout background due to the H-2K^K phenotype of these mice which would automatically delete the B cell compartment. However, this problem did not apply with the HyHEL10 transgene. Analysis of MD4 x LFA-1$^{-/-}$ mice revealed that their B cells have a

reduced capacity both to form tight contacts and to establish a synapse in the presence of ICAM-1, thus confirming that the direct interaction of LFA-1 with ICAM-1 is responsible for promoting these two events (Figures 4C and 5B).

The LFA-1/ICAM-1 Interaction Facilitates B Cell Triggering under Limited Antigen Conditions

To assess whether the enhancement in antigen recognition paralleled more efficient B cell activation, naive B cells were settled onto lipid bilayers loaded with different concentrations of antigen and ICAM-1. After 24 hr, activation was monitored by measuring the proportion of B cells which had upregulated both CD86 and CD69 (Figure 6 and data not shown). The results indicate that synapse formation and B cell activation exhibit a broadly similar dependence on the presence of ICAM-1. At high antigen density, more than 90% of B cells are activated.
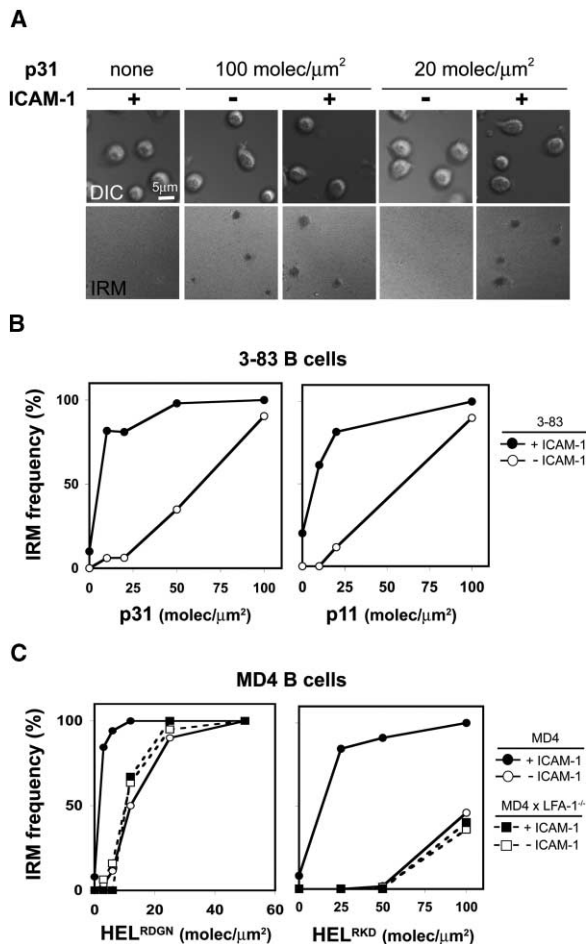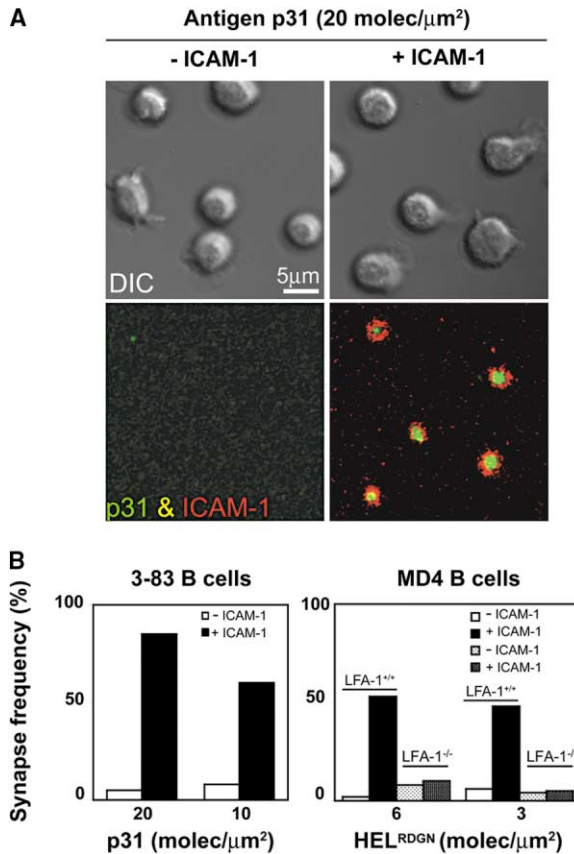
A

B

C

A

B

Figure 5. LFA-1/ICAM-1 Interaction Facilitates Synapse Formation at Low Antigen Density

(A) Synapse formation of 3-83 B cells in contact with membranes containing p31 antigen (green) at 20 mole/μm^2, in the presence or absence of ICAM-1 (red) at 170 mole/μm^2. DIC and fluorescence-merged images are shown.
(B) Naive 3-83 B cells or MD4 B cells were evaluated in the capacity to form a synapse on membranes loaded with p31 ($K_A = 6.5 \times 10^7$ M^{-1}) or HEL^{RDGN} ($K_A = 5 \times 10^8$ M^{-1}), respectively, in the presence or absence of ICAM-1. After 20 min, images were collected at each of the specified densities and the frequency of synapse was determined. A similar analysis was performed using naive MD4 $\times$ LFA-1$^{-/-}$ B cells on membranes loaded with HEL^{RDGN} ($K_A = 5 \times 10^8$ M^{-1}). Data are representative of four different experiments.

Figure 4. LFA-1/ICAM-1 Interaction Increases the Adhesion of B Cells to the Target Membrane

(A) DIC and IRM images of 3-83 B cells settled onto planar lipid bilayers in the presence or absence of ICAM-1 (170 mole/μm^2) and/or p31 antigen (0 mole/μm^2, left panels; 100 mole/μm^2, two center panels; and 20 mole/μm^2, two right panels). IRM shows a contact as a dark gray signal.
(B) Naive 3-83 B cells were evaluated in their capacity to form tight contacts on membranes loaded with p31 ($K_A = 6.5 \times 10^7$ M^{-1}) and p11 ($K_A \approx 7 \times 10^6$ M^{-1}) in the presence or absence of ICAM-1. After 5 min, images were collected at each of the specified densities, and the number of cells showing tight contacts was determined.
(C) A similar analysis was performed using in this case either naive MD4 (solid line) or MD4 x LFA-1$^{-/-}$ (dashed line) B cells on membranes loaded with HEL^{RDGN} ($K_A = 5 \times 10^8$ M^{-1}) and HEL^{RKD} ($K_A \approx 3 \times 10^6$ M^{-1}). Data are representative of three different experiments.

However, when density was limited, a higher proportion of B cells were activated only when ICAM-1 was included on the membranes. Furthermore, MD4 x LFA-1$^{-/-}$ B cells confirm that the cellular activation is not only due to a difference in membrane composition but also as a result of LFA-1/ICAM-1 interaction. Thus, under conditions of limited antigen, LFA-1/ICAM-1 facilitates B cell activation.

Discussion

In this study we have found that ICAM-1 participates in the B cell immunological synapse where it is recruited by LFA-1 and segregated from the BCR and antigen to the periphery. Unlike in T cells, a functional B cell synapse can be formed without adhesion molecules when the avidity of the B cell for the antigen exceeds a certain threshold. This offers the possibility of establishing the contribution of adhesion to synapse formation, which is not possible with T cells where adhesion is integral to antigen recognition. Whenever ICAM-1 is present, LFA-1/ICAM-1 and BCR/antigen segregate into the pSMAC/cSMAC pattern of a mature immunological synapse. Through this process of mature synapse formation, the inclusion of ICAM-1 in the synapse decreases the B cell avidity threshold by at least 10-fold. Thus, ICAM-1 enhances contact formation when the avidity of the B cell for the antigen is low, demonstrating a functional synergy between LFA-1 activity and BCR signaling.

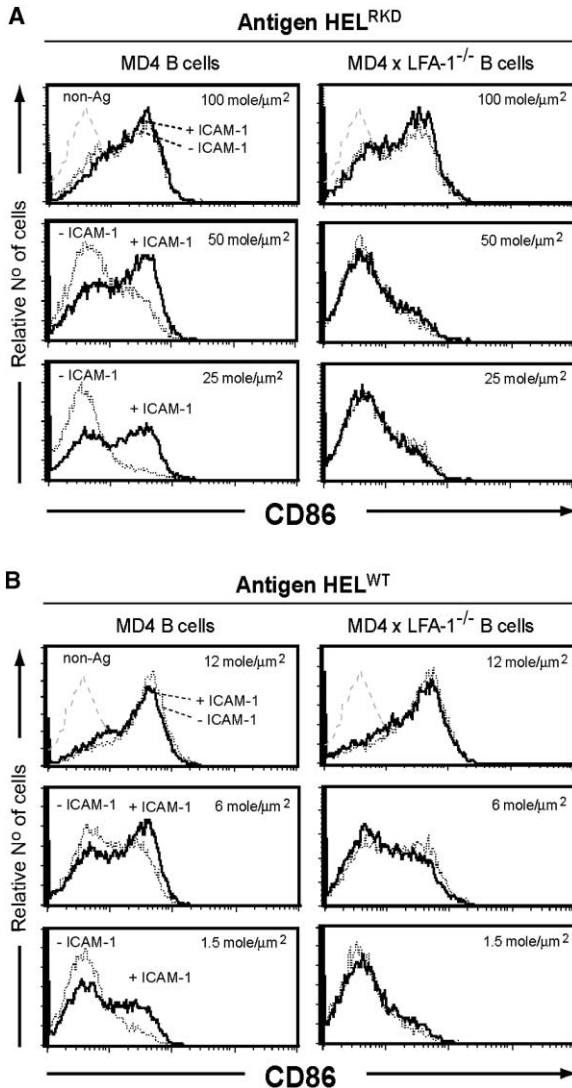These conclusions have been based on the analysis

A

Antigen HEL^RKD

B

Antigen HEL^WT

Figure 6. LFA-1/ICAM-1 Lowers the Threshold of B Cell Activation

Comparison of the activation of splenic B cells from MD4 or MD4 × LFA-1$^{-/-}$ mice following coculture with membranes either in the presence or absence of ICAM-1 and decreasing antigen densities. Activation was monitored by the upregulation of CD86. The results are shown as representative flow cytometry profiles of B cells that were stimulated with decreasing densities of either HEL^{RKD} (K$_A$ ≈ 3 × 10^6 M^{-1}) and HEL^{WT} (K$_A$ = 5 × 10^{10} M^{-1}) in the presence (solid line) or absence (dotted line) of ICAM-1 (170 mole/μm^2) for (A) and (B), respectively.

of splenic naive B cells from two different transgenic systems that allowed us to compare and contrast our observations over a wide range of physiological affinities. Our findings were also supported by the use of LFA-1-deficient B cells that verified the importance of LFA-1 as the functional receptor for ICAM-1 in this system. Our experiments were mainly based on an in vitro model system in which the interaction of naive B cells was analyzed with APCs and artificial lipid bilayers. While these in vitro studies have limitations, they allow us to dissect the role of LFA-1 in B cell activation. In vivo studies cannot provide such quantitative information as the contribution of this adhesion pair may be masked by other pathways which may in turn obscure the contribution of LFA-1.

The demonstration that, upon membrane antigen recognition, BCR and LFA-1 in naive B cells can be compartmentalized to form the cSMAC and pSMAC, respectively, is further evidence that the immunological synapse is a general molecular structure for recognition. The overall structure of the B cell synapse described in our present study appears to be very similar to the one originally reported for CD4 T cells (Grakoui et al., 1999; Krummel et al., 2000; Monks et al., 1998), CD8 T cells (Potter et al., 2001; Stinchcombe et al., 2001), and NK cells (Davis et al., 1999).

A remarkable feature of B cells is the range of affinities that the BCR must sense during the course of an immune response. Unlike T cells or NK, whose ligands are generally in the low-affinity range (van der Merwe and Simon, 2003), B cells are unique in that they must also sense very high-affinity interactions. Here we show that, over the whole spectrum of affinities analyzed (from 10^6 M^{-1} to 10^{11} M^{-1}), the compartmentalization of BCR and LFA-1 into cSMAC and pSMAC, respectively, was preserved. When the affinity of the BCR/antigen interaction was decreased, the density of antigen required for synapse formation was increased. Thus, the critical parameter is related to the total avidity of a B cell to a target membrane. However, if the affinity of the BCR for antigen was below 10^6 M^{-1}, we were unable to induce a synapse formation or show any signs of ICAM-1 aggregation, pointing toward an affinity threshold at which increasing density can no longer compensate. Once the affinity and density thresholds are exceeded, the amount of ICAM-1 that is recruited to the pSMAC is constant.

The formation of the immunological synapse has been modeled as a biophysical process dependent on the specific kinetic parameters of ligand binding by TCR and integrins, receptor mobility, and the mechanical properties of the membranes in which the molecules reside (Qi et al., 2001). This model is able to successfully predict the stages of immunological synapse formation observed experimentally. However, this model is mainly based on experimental data obtained from TCR/MHC kinetics and KIRs, where the affinity of the interaction is generally low. In some cases, NK cells show an inverse formation of cSMAC and pSMAC, which is interpreted as a consequence of the fast kinetics of this interaction. On that basis, it has been suggested that the formation of the immunological synapse is confined only to a narrow window of K$_{on}$/K$_{off}$ values (Lee et al., 2002a, 2002b). Predictions of this model fit well with data for CD4$^+$ T cell activation, but not CD8$^+$ T cell activation. Surprisingly, we found that the B cell immunological synapse can be formed over several orders of magnitude of K$_{off}$. This suggests that such a model may not account for the mechanism of B cell synapse formation. While structural differences among BCR, TCR, and KIRs might account for the difference in behaviors, our results provide a unique set of data on which such a model could be extended.

Strikingly, the interaction of B cells with their targets was accompanied by the formation of a docking structure, which appears to be formed at the site of contact. This cup-like structure shows remarkable similarities with the endothelial docking structures between PBL

with endothelial cells (Barreiro et al., 2002; Carman et al., 2003; Wojciak-Stothard et al., 1999). Interestingly, T cells and monocytes appear to take advantage of this structure to transmigrate through endothelial cell monolayers. We found that B cells can move several microns on the surface of the APC while maintaining their synapse. The velocity of this movement is measured in μm/hr and therefore is much slower than the migration of B cells in intact lymph nodes (Miller et al., 2002). A similar proposal was made previously for T cell migration over the surface of APC (Valitutti et al., 1995). However, an issue that remains to be addressed is whether such movement is B cell or target cell driven. Most importantly, it is not even clear whether a nomadic synapse could be of any benefit to a B cell. Nevertheless, we suggest that by moving its synapse, a B cell might increase the efficiency and amount of antigen gathered. One attractive possibility is that the formation of a mature B cell synapse may constitute a platform from which antigen extraction is facilitated, as well as provide other signals that may affect the fate of the B cell upon stimulation. Thus, by these processes, a B cell would favor its chances of being selected during affinity maturation.

Little is known about the function of LFA-1 on B cells. Studies involving LFA-1-deficient mice revealed a defect in B cell trafficking into peripheral lymph nodes, as well as accumulation in the bone marrow (Berlin-Rufenach et al., 1999). More recently, it has also been shown that LFA-1 binding to ICAM-1 is involved in the localization of marginal zone B cells, as well as the transit from the marginal zone into white pulp cords (Lo et al., 2003; Lu and Cyster, 2002). While these studies demonstrate a role for LFA-1 in B cell trafficking and localization, its function in B cell activation has not been analyzed in great detail. Our in vitro results demonstrate that, at low antigen densities, LFA-1 can help a B cell to adhere, form a synapse, and become activated. These observations predict that the B cell response in LFA-1-deficient animals would be reduced.

While LFA-1 was essential at low antigen concentrations, the frequency of synapse and B cell activation was the same at high antigen density in the presence or absence of ICAM-1. These observations nicely correlate with the work of Wulfing and Davis in T cells, which demonstrates that ICAM-1 may increase the chances of a productive calcium signal at low amounts of peptide and result in increased activation (Wulfing et al., 1998). Similarly, LFA-1 facilitates T cell activation by lowering the amounts of antigen necessary for T cell activation (Bachmann et al., 1997). Furthermore, it has been demonstrated that ICAM-1 provides a selective boost to signaling for differentiation among CD4$^+$CD8$^+$ cells (Lucas and Germain, 2000). Thus, LFA-1 appears to have an impact in lymphocyte activation when the total avidity of the antigen receptors is low.

A pertinent observation is that the LFA-1/ICAM-1 interaction is remarkable in inducing B cell attachment to a target membrane. This effect appears particularly strong when avidity of the membrane for the BCR and antigen density is low. A likely explanation is that, on the surface of naive B cells, LFA-1 is in a low-avidity state, unable to support sustained B cell adhesion. After initial contact with antigen on the membrane, a small amount of engaged BCR may produce an adequate level of signal to switch LFA-1 into its high-avidity state, but not sufficient to produce a visible synapse or to fully activate a B cell. Such a mechanism is supported by the studies of Rock et al., which showed that, after BCR crosslinking, LFA-1 binding to ICAM-1 is increased (Dang and Rock, 1991). In accordance with these findings, our observations show that all B cells will achieve tight contact with membranes containing ICAM-1 only if their BCRs are previously engaged. Such an increase in attachment could, therefore, facilitate antigen aggregation and synapse formation, leading to increased activation.

Other adhesion molecules may play a similar role to the one we describe here for LFA-1. A possible candidate would be VLA-4, whose ligand VCAM-1 is highly expressed on FDCs. The interaction of VLA-4/VCAM-1 has also been involved in promoting the adhesion of B cells to FDCs (Koopman et al., 1991). Furthermore, recent studies show that LFA-1 and VLA-4 play a redundant role in the adhesion of marginal zone B cells within the splenic marginal zone (Lo et al., 2003; Lu and Cyster, 2002). Indeed, we show here that naive B cells are able to make short-term LFA-1-independent contacts with their targets in the absence of antigen. These B cells formed indistinguishable conjugates from those observed in the presence of antigen. However, no polarization of the BCR was observed, and interactions with the same target were short term. Analogous conjugates were also formed in the absence of ICAM-1, which indicates that other adhesion molecules might be involved.

An important implication of membrane antigen recognition is that the cell surface, or context in which antigen is seen, may be involved in the recruitment of other accessory receptors that may modulate the threshold of B cell activation. It seems conceivable that multiple mechanisms may exist to assist the BCR in self-/non-self-discrimination. It has been shown that antigen presented on the surfaces of professional APCs promotes B cell activation through CD21, indicating that complement deposition on the surface of FDC plays a role in enhancing antigen recognition (Tew et al., 1997). On the other hand, we have recently shown that the presence of α2,6-sialoconjugate on the surface of target cells dampens B cell activation (Lanoue et al., 2002). This may be achieved by recruiting CD22, an inhibitory receptor, which specifically recognizes this sialoglycoconjugate. Thus, the context in which a B cell sees antigen is likely to be important in determining which accessory molecules are recruited and therefore biasing B cells toward activation or tolerance.

At this point our understanding of B cell activation by membrane antigens is only limited, but on the basis of our results we can postulate a model by which we can divide the sequence of events into four steps. First, the B cell and the APC make contact, probably through an adhesion mechanism. In the second phase, which may overlap with the first, some antigen engagement might locally alter the cell surface oligomeric structure of the BCR (Reth, 2001; Schamel and Reth, 2000) generating a signal which could increase the state of adhesion of the B cell, leading to enhanced attachment to the target. If the signal is not high enough, the B cell will simply detach; however, if the signal received is high, a visible synapse will become apparent. According to the type

of signal generated, the B cells may become activated, differentiate, or eventually become anergic (Goodnow, 1996). Finally, in step four, the B cell will acquire antigen from the synapse to present to T cells.

The context in which antigen is detected by a B cell might be critical in determining which coreceptors may or may not be recruited, thereby amplifying or inhibiting the signal by the BCR. The coordinated function of LFA-1 and the BCR is going to play a role in determining the fate of the B cell, particularly at low antigen doses. Further understanding of the early cellular and molecular events of membrane recognition will help us to comprehend B cell activation, anergy, or autoimmunity.

Experimental Procedures

Mice and Splenic B Cell Purification

Two different BCR transgenic systems have been used, 3-83 mice (H-2K^k-specific BCR; Russell et al., 1991) and MD4 mice (Hen Egg Lysozyme (HEL)-specific BCR; Goodnow et al., 1988). MD4 mice were crossed with LFA-1$^{-/-}$ mice (generous gift from Dr. N. Hogg, London, UK; Berlin-Rufenach et al., 1999) to obtain the MD4 x LFA-1$^{-/-}$ strain. Splenic naive B cells were isolated by negative selection, treating total splenocytes after a Lympholyte step (Cedarlane Laboratories, Ontario, Canada) with Dynabeads mouse pan-T (Dynal Biotech ASA, Oslo, Norway). This procedure enriched to 95%–98% B cells.

Time-Lapse Video Microscopy and Time-Lapse Confocal Microscopy

Murine L cells fibroblasts were used as APCs for 3-83 B cells, as they express the class I antigen H-2K^k on their surface. L cells were transfected with ICAM-1-GFP construct (generous gift of Dr. M.M. Davis, Stanford, CA).

Time-lapse video microscopy was performed using a Nikon Diaphot 200 inverted microscope equipped with a CCD camera (Hamamatsu Orca). ICAM-1-GFP APCs were split on 35 mm glass-bottom dishes (MatTek Corporation, Ashland, MA). The dish was placed on a 37°C heating stage, and 2×10^6 naive B cells were added. The assay was done in RPMI 10% FCS, 50 mM HEPES medium. Phase contrast frames were captured with a 40× dry-objective. The tracking analysis was done with Kinetic Imaging AQM software.

For time-lapse confocal microscopy, ICAM-1-GFP APCs were split on 4 mm coverslips. The coverslip was assembled in FCS2-flow chamber (Bioptechs), and 2×10^6 3-83 B cells, previously stained with Cy5-conjugated nonblocking Fabs anti-IgM (Jackson Immunology Research Laboratories), were injected. The assays were done in PBS 0.5% FCS, 1 gr/l D-glucose, 2 mM Mg^{2+}, 0.5 mM Ca^{2+}, at 37°C. Confocal series of DIC and fluorescence images were simultaneously obtained at the specified intervals in each case, with a 63× oil immersion objective. Imaging was carried out on a Zeiss Axiovert LSM 510-META inverted microscope, and the image analysis, 3D reconstruction, and movie assembly were performed using LSM 510 software (Zeiss, Germany).

Adhesion Assay

DCEK or EKAM1.5 murine cell lines were plated at confluence on 96-well plates. $3–5 \times 10^5$ purified B cells were added per well and incubated for 30 min at 37°C. Then, wells were washed three times with warm assay buffer (PBS 0.5% FCS, 2 m Mg^{2+}, 0.5 mM Ca^{2+}, 1 gr/l D-glucose). The remaining B cells were recovered by treatment with dissociation buffer (Sigma) and counted.

3-83 Peptide Ligands and Recombinant Lysozymes

Four different monobiotinylated peptides were obtained by solid synthesis: p31 (NH$_2$-SGSGHDWRSGFGGFQHLCCSGS-COOH), p11 (NH$_2$-SGSGGMNWNWLQAHTSG SG-COOH), p5 (NH$_2$-SGSGLMNT GGYQSLLPSGS-COOH) (Kouskoff et al., 1998), and p0 or null (NH$_2$-SGSGPRLDSAKEIMASGSC-COOH). The lysozymes utilized in this study were: HEL^{WT}, for HEL wild-type; HEL^{RDGN}, with amino acid positions 21 101, 102, 103 mutated to alanine; HEL^{KD}, with aa 97,

101; and HEL^{KD}, with aa 21, 97, 101. The different HEL mutants (Batista and Neuberger, 1998; F.D.B. and M.S. Neuberger, unpublished data) were expressed as His-tagged proteins in J558L cell line and purified from the supernatant by Ni-NTA agarose columns (QIAGEN).

Surface Plasmon Resonance

The interaction between p31, p11, p5, and 3-83 mAb was analyzed by the BIAcore biosensor system, BIAcore 2000 (BIAcore, Uppsala, Sweden), as described previously (Batista and Neuberger, 1998). In brief, the different biotinylated peptides were captured in SA sensor chips (BIAcore). Then, various concentrations of mAb 3-83 were injected at a flow of 5 μl/min. The sensorgrams of the interactions between the negative control p0 and the 3-83 mAb were subtracted from the interactions of p31, p11, and p5 and mAb 3-83. All the binding assays were performed in HEPES-buffered saline (pH 7.4) (BIAcore).

Expression of GPI-Linked Murine ICAM-1

The extracellular domain of murine ICAM-1 was amplified by PCR with cDNA obtained from murine 3T3 NIH fibroblasts as a template, the sense 5′-CGGGATCCGCCCTGCAATGGCTTCAACC-3′ primer, and the antisense 5′-GCGAATTCCACTACCTGATTGAGAGTGGTA CAGTACTGTC-3′ primer. The fragment obtained was cloned in BamH1/EcoRI restriction sites on pcDNA3 expression vector. The GPI domain coding sequence (Coyne et al., 1993) was obtained by annealing of the sense 5′-CGGAATTCTATCAGCTGGTACTACCAC TACGACCACTACAACGCTGTTGCTATTACTCCTATTGCTATTATTG CTCCTGTAGGATATCGA-3′ primer and the antisense 5′-GAGAT ATCCTACAGGAGCAATAATAGCAATAGGAGTAATAGCAACAGCGT TGTAGTGGTCGTAGTGGTAGTACCAGCTGATAGAATTCCG-3′ primer, and was added to the ICAM-1 extracellular domain by digestion and cloning into EcoR1/EcoRV restriction sites of pcDNA3 expression vector. The GPI-linked ICAM-1 construct was transfected in murine L fibroblasts, and positive clones were selected in the presence of geneticin.

Planar Lipid Bilayers

The GPI-linked ICAM-1 protein was purified from transfected L cells as previously described (Bromley et al., 2001). In brief, the protein was solubilized in 1% Triton X-100 buffer and captured on an anti-ICAM-1 BE29 antibody (ATCC)-coupled sepharose column. The protein was labeled with Alexa Fluor 532 (Molecular Probes) and eluted at low pH. The purified labeled GPI-linked ICAM-1 was reconstituted into liposomes by detergent dialysis with 0.4 mM egg phosphatidylcholine (egg-PC) (Avanti Polar Lipids, Inc.).

Planar lipid bilayers were formed as previously described (Grakoui et al., 1999). Alexa Fluor 532-labeled GPI-linked ICAM-1 liposomes and/or biotinylated lipids were mixed with 1,2-dioleoyl-PC (DOPC) lipids (Avanti Polar Lipids, Inc.) at different ratios to get the molecular density required. The chambers were blocked with PBS 2% FCS, followed by the antigen loading in PBS 0.5% FCS. The antigens for 3-83 B cells were loaded in the bilayers as mono-biotinylated peptides, previous incubation with Alexa Fluor 488-conjugated avidin (Molecular Probes). The different HEL antigens were loaded through monobiotinylated anti-HEL antibody F10 (Schwarz et al., 1995), anchored to the membrane by Alexa Fluor 488 avidin. The B cells were injected into the warmed (37°C) chamber at time zero, then the flow was stopped during the experiment. The assays were done in PBS 0.5% FCS, 2 mM Mg^{2+}, 0.5 mM Ca^{2+}, 1 gr/l D-glucose, at 37°C. Confocal fluorescence and DIC images were simultaneously obtained at the specified times. The contact of B cells with the planar lipid bilayer was visualized by interference-reflection microscopy (IRM). Images were acquired on a Zeiss Axiovert LSM 510-META inverted microscope with a 63× oil immersion objective and analyzed by LSM 510 software (Zeiss, Germany).

The quantification of the number of ICAM-1 or antigen molecules recruited in the synapse was calculated as accumulated density (molecules/μm^2) = [intensity in the selected area (fluorescence units/μm^2) − intensity in neighboring area (fluorescence units/μm^2)]/ specific activity (fluorescence units/molecule). The total number of molecules recruited was obtained multiplying the accumulated density (molecules/μm^2) by the selected area (μm^2) in each cell or time

point, which is given as the area where the aggregated molecules exceed the density in the lipid bilayer. The specific activities of GPI-linked ICAM-1 and of antigen in the planar lipid bilayers were determined by immunofluorometric assay using anti-ICAM-1 or anti-κ antibodies (against F10), respectively. Standard values were obtained from microbeads with different calibrated binding capacities of mouse IgG on their surface (Quantum Simply Cellular, Bangs Laboratories, Inc., IN). Identical specific activity was considered to estimate the amount of antigen in both antigens systems, since the same Alexa Fluor 488-conjugated avidin was utilized.

B Cell Activation Assays
Planar lipid bilayers with biotinilated-lipids and/or Alexa Fluor 532-labeled GPI-linked ICAM-1 were formed on 6 mm diameter Multiwell Chambered Coverslip (Molecular Probes) by incubation at RT during 20 min. Then, wells were blocked with PBS 2% FCS, followed by the antigen loading as previously described for the flow chambers. 0.25×10^6 B cells in RPMI 10% FCS were added per well. B cells were collected after 20 hr at 37°C and stained with Cy-Chrome-conjugated anti-mouse CD45/B220 mAb, PE-labeled anti-mouse CD69 mAb, and FITC-conjugated anti-mouse CD86 mAb (BD Biosciences). The samples were analyzed in a FACScalibur cytometer (BD Bioscience).

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